Purification and Properties of the Aldehyde Oxidases from Hog and Rabbit Livers*

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SUMMARY

The aldehyde oxidases from rabbit and hog livers have been purified to homogeneity by modifications of and additions to previously published procedures. The final preparations were judged to be homogeneous on the basis of ultracentrifugal, electrophoretic, chromatographic, spectral, and kinetic criteria. A number of physical and kinetic properties of the homogeneous enzymes have been determined. The homogeneous enzymes have been shown to catalyze the oxidation of N'-methyl nicotinamide to both N'-methyl-2-pyridone-5-carboxamide and N'-methyl-4-pyridone-3-carboxamide in a ratio (2-pyridone to the 4-pyridone) of 100 for the rabbit enzyme and 3.8 for the hog enzyme. The ratios of products formed by these enzymes at each step in their purifications were constant. The ratios of the pyridone products obtained on the oxidation of several N'-substituted analogues of N'-methyl nicotinamide by these aldehyde oxidases have been found to shift in favor of the 4-pyridone as the bulk of the N'-substituent was increased. A general mechanism of action of aldehyde oxidases is proposed which accounts for the different pyridone ratios and the unique kinetic properties associated with the different forms of the enzyme which have been found in a number of different mammalian livers.

N'-Methylnicotinamide is oxidized to 4-pyridone and 2-pyridone in a number of mammals (1-11). Xanthine oxidase from both hog liver (3), bovine milk (12), and rabbit liver aldehyde oxidase (1) is known to catalyze the oxidation of N'-methyl nicotinamide to the 2-pyridone. Since the conditions under which xanthine oxidase can oxidize the oxidation of N'-methyl nicotinamide are not those generally considered to be physiologically, it is likely that aldehyde oxidase is generally the most significant catalyst of the formation of the 2-pyridone in vivo.

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The abbreviations used are: 4-pyridone, N'-methyl-4-pyridone-3-carboxamide; 2-pyridone, N'-methyl-2-pyridone-5-carboxamide.

(13). Man and the rat are known to be exceptions to this rule (13). Although genetic data suggested aldehyde oxidase is the enzyme responsible for the oxidation of N'-methyl nicotinamide to the 4-pyridone (14), this point has not been unequivocally proven through examination of a homogeneous aldehyde oxidase. Although it is not usual for an enzyme to catalyze the formation of two alternate products from a single substrate, the properties of aldehyde oxidase are compatible with such an idea.

Since aldehyde oxidase can oxidize a large number of nitrogenuous compounds of widely differing structures (15, 16), it would not be an unreasonable extension of its lack of specificity to conclude it might catalyze the oxidation of N'-methyl nicotinamide at two alternate positions. In order to test this hypothesis, the purification and characterization of the hog and rabbit liver aldehyde oxidases was undertaken.

EXPERIMENTAL PROCEDURE

Materials

N'-Methylnicotinamide, N'-propyl[14C]nicotinamide, and N'-2,6-dichlorobenzyl[14C]nicotinamide were synthesized from [7-14C]nicotinamide (New England Nuclear) by the method of Holman and Wiegand (17). The compounds were purified by paper chromatography or crystallization or both. The unlabeled compounds were prepared in a similar fashion. N'-Methylnicotinamide iodide was converted to the chloride form by passage through a Dowex 1-X8 chloride (50 to 100 mesh) column. The 2-pyridone was obtained from K and K Laboratories or was synthesized by the method of Pullman and Colowick (18). Catalase (EC 1.11.1.6) was purchased from Worthington, lactic acid dehydrogenase (EC 1.1.1.27) from Boehringer, crystallized lyophilized bovine serum albumin from Sigma, and DEAE-microgranular cellulose from Whatman. Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) and riboflavin-5'-phosphate were obtained from Calbiochem. Analytical grade reagents were used throughout. Rabbit livers were obtained from a local rabbitry and hog livers from Hill Top Packers (Roseville, California). Calcium phosphate gel was prepared by the method of Singer and Kearney (19). The final neutralization step was omitted. Alumina Cγ gel was prepared as previously described (20).

Methods

Assay of Aldehyde Oxidase—The increase in optical density at 300 nm which accompanies the oxidation of N'-methyl nicotinamide to the 2- and 4-pyridones was used as the basis for the assay.
of enzyme activity. Reaction mixtures were set up in a cuvette having a 1-cm light path. In the assay of the rabbit liver enzyme they contained the following in a final volume of 1.0 ml, N\textsuperscript{\textdagger}methyl nicotinatamine, 5.0 µmoles; potassium phosphate buffer, 70.0 µmoles, pH 7.8; bovine serum albumin, 1.25 mg; estalase, 12 µg; and a suitable amount of enzyme and water. One unit of enzyme activity was defined as the amount of enzyme which oxidized 1 µmole of N\textsuperscript{\textdagger}methyl nicotinatamine per min at 25\textdegree. For the assay of the hog liver enzyme the reaction mixtures contained the following in a final volume of 1.0 ml, N\textsuperscript{\textdagger}methyl nicotinatamine, 5.0 µmoles; glycine buffer, 160 µmoles, pH 9.5; allopurinol, 0.03 µmoles; and a suitable amount of enzyme and water. Allopurinol was included in order to inhibit the oxidation of N\textsuperscript{\textdagger}methyl nicotinatamine by the xanthine oxidase which was present in enzyme fractions. This was not a problem in the rabbit system because of the higher specific activity of the rabbit aldehyde oxidase. The presence of allopurinol had no effect on the homogeneous preparation. The optical density change was followed in a Cary 14 recording spectrophotometer equipped with a 0 to 0.2 optical density slide wire. One unit of enzyme activity was defined as the amount of enzyme which oxidized 1 µmole of N\textsuperscript{\textdagger}methyl nicotinatamine per min at 25\textdegree. Specific activity for both enzymes was defined as the number of enzyme units per mg of protein. A molar extinction coefficient of 4.17 \times 10\textsuperscript{3} was used to convert optical density changes at 300 nm to moles of pyridones formed.

**Determination of Protein Concentration**—The protein concentration of impure enzyme fractions was estimated by the biuret method (21). Protein determination on all purified hog liver aldehyde oxidase fractions was based on the method of Lowry et al. (5). Protein determinations on all purified rabbit liver aldehyde oxidase fractions were based on absorbance at 280 nm and related to dry weight by using E\textsubscript{280}m\textsubscript{m} = 12.4. This value was obtained by measuring the absorbance of an enzyme sample which had been exhaustively dialyzed against deionized water and then dried to constant weight in a vacuum oven at 106\textdegree.

**Determination of Pyridone Ratios**—For the rabbit enzyme the reaction mixtures were set up in a conical centrifuge tube and contained the following in a final volume of 0.060 ml: catalase, 3 µg; bovine serum albumin, 63 µg; potassium phosphate buffer, pH 7.8, 3 µmoles; and either N\textsuperscript{\textdagger}methyl[7-\textsuperscript{\textdagger}C] nicotinatamine, 0.274 µmole (2 µCi per µmole), or N\textsuperscript{\textdagger}propyl[7-\textsuperscript{\textdagger}C] nicotinatamine, 0.307 µmole (0.008 µCi per µmole), and appropriate volumes of water and enzyme. Incubations were carried out either at room temperature or 37\textdegree for periods of from 1 to 4 hours. Ratios were not affected by temperature or the time of incubation. Reactions were terminated by placing the reaction vessels in a boiling water bath for 2 min. Insoluble protein was removed by centrifugation and the reaction products were separated and determined as described previously (2).

For the hog enzyme the determination of the pyridone ratios was carried out in reaction mixtures containing the following in a final volume of 0.050 ml: glycine buffer, 5 µmoles, pH 9.5; allopurinol, 0.015 µmole; N\textsuperscript{\textdagger}methyl[4-\textsuperscript{\textdagger}C] nicotinatamine, 0.274 µmole, 2 µCi per µmole; and appropriate amounts of water and enzyme. Ratios of products formed in comparative studies of substrate analogues were determined in reaction mixtures containing the following in a final volume of 0.006 ml: glycine buffer, 2 µmoles, pH 9.5; N\textsuperscript{\textdagger}methyl[7-\textsuperscript{\textdagger}C] nicotinatamine, 0.274 µmole (2 µCi per µmole), or N\textsuperscript{\textdagger}propyl[7-\textsuperscript{\textdagger}C] nicotinatamine, 0.307 µmole (0.008 µCi per µmole), and appropriate amounts of water and enzyme. All other operations were carried out as previously described (2).

**Assay of Xanthine Oxidase**—Xanthine oxidase activity was determined spectrophotometrically by following the change in optical density at 295 nm which accompanies the oxidation of xanthine to uric acid (12).

**Ultracentrifugal Analysis**—Sedimentation velocity and sedimentation equilibrium analyses were made using a Spinco model E ultracentrifuge. Protein samples were dialyzed for 24 hours against Tris-HCl buffer, pH 7.8, 10 mM, containing NaCl, 100 mM, EDTA, 0.1 mM, and cysteine, 5 mM prior to being run. The dialyzed enzyme was diluted to the appropriate concentrations with dialysate. Sedimentation coefficients were converted to s\textsubscript{20,w} and extrapolated to zero protein concentration according to the procedure described by Schachman (22). In sedimentation equilibrium experiments an initial overspeed velocity of 17,680 rpm was applied for 120 min and equilibrium obtained in an additional 18 to 22 hours at 12,600 rpm.

**FAD Analysis**—Estimation of flavin adenine dinucleotide was based on the method of Burch (23). Fluorescence was determined with a Farrand Spectrofluorometer. Riboflavins standards were prepared using a molar extinction coefficient of 450 nm of 12.2 \times 10\textsuperscript{3} (24). Fluorescence was measured at 535 nm after excitation at 450 nm. The amount of FAD obtained was related to the amount of enzyme, from which it was derived, through the extinction coefficient of the enzyme cited earlier. Calculations of the number of moles of FAD per mole of enzyme were based on a molecular weight of the enzyme of 270,000.

**Metal Analysis**—The iron content of the enzyme was determined colorimetrically by the o-phenanthroline method (25). The samples were clarified by centrifugation immediately before the determination of their absorbance at 511 nm. Molybdenum estimations were performed by the tolulene-dithiol method of Clark and Axley (26) with a slight modification. The modification involved the use of 1.0 ml of isoamylacetate in the extraction procedure. Calculation of the number of metal atoms per mole of enzyme was based on the previously cited extinction coefficient and a molecular weight of 270,000.

**Column Chromatography**—Columns (2.5 \times 20 cm) were prepared from microgranular DEAE-cellulose which had been washed with 0.5 mM NaOH, 0.5 mM HCl, deionized water, and a 0.01 M Tris-HCl bufier, pH 8.3, 10\textsuperscript{\textdagger}M EDTA, 0.005 M cysteine. A flow rate of approximately 1 ml per min was used for chromatography of the enzyme and 15-ml fractions were collected.

**Electrophoresis**—Analytical acrylamide gel disc electrophoresis was performed in a model 12 Canaleo disc electrophoresis apparatus. The gel system used in routine work was that of Ornstein and Davis (27). The method of Hedrick and Smith (28) was employed in the disc gel molecular weight analysis. A copper wire was imbedded in the gel to mark the dye front. Protein bands were stained with Amido schwarz. Aldehyde oxidase bands were also detected using a substrate specific activity stain. The enzyme staining solution contained the following in a final volume of 3.0 ml N\textsuperscript{\textdagger}methyl nicotinatamine, 5.0 µmoles; nitroblue tetrazolium, 0.75 mg; and potassium phosphate buffer, pH 8.0, 0.29 mM. Disc gels were immersed in the staining solution immediately upon completion of electrophoresis. After adequate color development, the gels were transferred to and stored in 7% acetic acid. Preparative electrophoresis was performed at a temperature of 4\textdegree using a commercial instrument (Buchler Instruments, Inc., Fort Lee, N.J.). Samples (5 to 10 ml) containing 50 to 150 mg of protein in 3% sucrose were run at 45 ma in the Tris-phosphate system described in the Buchler Instruction Manual. Ammonium persulfate and riboflavin catalysts were replaced by riboflavin-5\textsuperscript{\textdagger}phosphate at a final
Purification Procedure for Rabbit Liver Enzyme

The procedure is the result of modifications of and additions to the method of Rajagopalan et al. (1). Only fresh livers from New Zealand White rabbits were used. Maximum yield of enzyme was dependent on the use of healthy well fed and mature animals. All operations were carried out at 0–5°C unless specified otherwise. A typical preparation was carried out using 200 g of rabbit liver.

Steps 1 to 3. Extraction and Heat Treatment, Ammonium Sulfate Precipitation, and Acetone Fractionation—These steps were, with the exception of some small modifications, identical to those previously published (1). The modifications were as follows: all buffers contained EDTA, 10⁻⁴ M; the homogenate was heated in 300- to 350-ml portions in 1-liter Erlenmeyer flasks for 11 min at 55°C, rather than 15 min at 55–60°C; the acetone fractionation was carried out at a protein concentration of 42 mg per ml and the protein fraction, which precipitated between 42 and 48% (rather than 43 and 50%) acetone, was retained.

Step 4. Calcium Phosphate Adsorption and Elution—The protein concentration of the solution from Step 3 was adjusted to 10 mg per ml by the addition of deionized water. Calcium phosphate gel (2 to 4 mg gel per mg of protein) was added to the diluted protein solution and the mixture was stirred in an ice bath for 15 min. The gel was collected by centrifugation and washed twice with deionized water for 15 min. The gel was then extracted four or five times by stirring for 15 min with 0.01 M potassium phosphate buffer, pH 7.8. After each extraction, the gel was removed by centrifugation and the eluates combined. The protein was concentrated by precipitation with ammonium sulfate, 0.60 saturation. The precipitate was collected by centrifugation and dissolved in 10 to 20 ml of 0.01 M Tris-HCl buffer, pH 8.2 containing 10⁻⁴ M EDTA and 0.005 M cysteine. The next step was carried out immediately.

Step 5. DEAE-cellulose Chromatography—The enzyme solution from the calcium phosphate gel step was dialyzed 4 to 5 hours against two changes of 100 volumes of 0.01 M Tris-HCl buffer, pH 8.3; EDTA, 10⁻⁴ M; and cysteine, 0.005 M. The dialyzed enzyme was applied to a column (9.5 × 90 cm) containing DEAE-microcellulose. The enzyme was eluted with a linear gradient of 600 ml of buffer, 0.01 M Tris-HCl, pH 8.3; EDTA, 10⁻⁴ M; cysteine, 0.005 M; and 600 ml of buffer made 0.3 M in NaCl. The fractions were scanned for absorption at 280 nm and assayed. The first large protein peak which was eluted from the column (Fractions 30 to 50) contained the bulk of the aldehyde oxidase activity. Considerable colored material and protein remained on the column. The fractions which contained enzymatic activity were combined and ammonium sulfate was added to 0.60 saturation. The precipitate was collected by centrifugation and dissolved in 5 to 15 ml of a 1:4 dilution of the Tris-phosphate concentrating gel buffer described in the Buchler Poly-Prep Apparatus Instruction Manual. The diluted buffer was made 0.005 M in cysteine and adjusted to pH 8.0 before dissolving the enzyme in it. The next step was usually carried out immediately.

Step 6. Preparative Acrylamide Electrophoresis—The enzyme solution from Step 5 was dialyzed 3 to 4 hours against the cysteine-containing diluted concentrating gel buffer which was described in the previous step. After dialysis the solution containing enzyme was made 3% in sucrose and put onto the Buchler Poly-Prep Apparatus; a typical run lasted about 7 hours. The bulk of the aldehyde oxidase activity was eluted in the first large protein peak. The run was terminated when the enzyme had been recovered; considerable protein remained on the gel at that time. The fractions containing enzyme at a constant specific activity were combined and concentrated by ultrafiltration to 5 to 15 mg per ml. The enzyme was usually stored at 4°C and protected from exposure to light. When the enzyme was stored in this manner or was quick frozen in a Dry Ice-acetone bath and stored at −70°C it was stable for at least 1 month. A summary of a typical purification is presented in Table I. The final specific activities of purified enzyme ranged from 1.02 to 1.91, with most preparations having a specific activity of about 1.6. Purified enzyme had an intense yellowish-gold to red-orange color in dilute or concentrated solutions, respectively.

Purification Procedure for Hog Liver Enzyme

Livers from 200-pound Chester White hogs were cut into thin slices, quick frozen between blocks of Dry Ice, and stored at −70°C until used. Unless otherwise specified all operations connected with the purification of the enzyme were performed at 0–5°C. A typical preparation was carried out using 200 g of liver.

Step 1. Extraction—The livers were thawed, minced, and homogenized in a Waring blender for 1 min with 3 volumes of

| Table I. Purification of rabbit liver aldehyde oxidase

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units</td>
<td>mg</td>
<td></td>
<td>%</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>1. Extraction and heat treatment</td>
<td>1945</td>
<td>395</td>
<td>22,560</td>
<td>0.0162</td>
<td>100</td>
<td>37.0</td>
<td>100</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄, 0–0.50</td>
<td>80</td>
<td>598</td>
<td>6,430</td>
<td>0.0585</td>
<td>98</td>
<td>54.4</td>
<td>115</td>
</tr>
<tr>
<td>3. Acetone, 0.42–0.48</td>
<td>52.7</td>
<td>254</td>
<td>1,600</td>
<td>0.158</td>
<td>70</td>
<td>6.84</td>
<td>19</td>
</tr>
<tr>
<td>4. Calcium phosphate gel</td>
<td>18.6</td>
<td>163</td>
<td>267</td>
<td>0.0777</td>
<td>42</td>
<td>0.000</td>
<td>0</td>
</tr>
<tr>
<td>5. DEAE-cellulose</td>
<td>9.7</td>
<td>81.8</td>
<td>62.6</td>
<td>1.31</td>
<td>22</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>6. Preparative electrophoresis</td>
<td>3.0</td>
<td>5.25</td>
<td>27.5</td>
<td>1.91</td>
<td>14.4b</td>
<td>109</td>
<td></td>
</tr>
</tbody>
</table>

a. Absorbance change per min.
b. Yields in this step depended upon contaminating protein which was sometimes present and ran close to and just ahead of the enzyme. If the contaminant was present in high amounts, overlap sometimes occurred and the yields of pure enzyme were reduced.
potassium phosphate buffer, 0.15 M, pH 6.8, 10^{-4} M EDTA. The homogenate was filtered through cheesecloth and centrifuged 40,000 \times g for 20 min; the precipitate was discarded.

Step 2. Heat Treatment—Portions (75 ml) of the clarified homogenate from the previous step were transferred to 250-ml Erlenmeyer flasks and heated to 55°C by placing the flasks in a 75-80°C water bath. After the clarified homogenate had reached 55°C, the flasks were placed in a 56°C water bath for 4 min. The homogenate was then cooled quickly to below 15°C by placing the flasks in an ice bath. During both the heating and cooling procedures the homogenate was stirred. The heat-treated homogenate was centrifuged at 40,000 \times g for 20 min and the precipitate discarded.

Step 3. Ammonium Sulfate Precipitation—Enough solid ammonium sulfate was added to the solution of heat-treated enzyme in order to bring it to 0.3 saturation. The solution was stirred for 10 min, the precipitate was removed by centrifugation, and the enzyme was precipitated by the addition of ammonium sulfate to 0.45 saturation. After stirring for 10 min, the precipitate was collected by centrifugation and dissolved in 50 ml of potassium phosphate buffer, 0.1 M, pH 7.8. The solution was adjusted to pH 7.8 with 2 M potassium hydroxide and clarified by centrifugation. The next step was carried out immediately.

Step 4. Acetone Fractionation—The acetone step was identical to that described for the rabbit enzyme except that the 40 to 47% acetone precipitate was collected and adjusted to pH 8.9 before clarification and storage. The enzyme was stable at this point when stored overnight in the refrigerator.

Step 5. Alumina Cy Adsorption and Elutions—The protein concentration of the solution from Step 4 was adjusted to 10 mg per ml by the addition of deionized water. Alumina Cy (1.5 mg of gel per mg of protein) was added to the diluted protein solution and the mixture was stirred in an ice bath for 10 min. The gel was collected by centrifugation and washed by stirring with successive portions of potassium phosphate buffer, 0.05 M, pH 7.8, 10^{-4} M EDTA until about 10% of the total activity was eluted. The washes were discarded and the gel was then extracted two or three times by stirring for 10 min with potassium phosphate buffer, 0.5 M, pH 7.8, 10^{-4} M EDTA. After each extraction, the gel was recovered by centrifugation and the eluates were combined. The protein was concentrated by precipitation with ammonium sulfate, 0.60 saturation. The precipitate was collected by centrifugation and dissolved in a small volume of 0.01 M Tris-HCl buffer, pH 8.2 containing 10^{-4} M EDTA and 0.005 M cysteine. The next step was carried out immediately.

Step 6. DEAE-cellulose—The DEAE-cellulose step was identical to that described for the rabbit enzyme (2). Fractions eluted from a (2.5 \times 25 cm) column were scanned for absorption at 280 nm and assayed. Although protein eluted in a broad band between Fractions 10 and 100, the aldehyde oxidase activity came off the column in a sharp peak (Fractions 45 to 55). The enzyme after this step was unstable to storage so the next step was always carried out immediately.

Step 7. Preparative Acrylamide Electrophoresis—The enzyme from Step 6 was carried through preparative acrylamide electrophoresis as described for the rabbit liver enzyme. A typical run lasted 9 to 10 hours. Unlike the case of the rabbit enzyme, the elution of the hog enzyme was preceded by a series of other proteins. After concentration the enzyme was quick frozen in a Dry Ice-acetone bath and stored at –70°C. The enzyme stored in this manner was stable for at least 2 months. Table II gives a summary of a typical purification. The specific activity of the purified enzyme was generally 110 to 120, however, two preparations with half this activity were obtained.

**RESULTS AND DISCUSSION**

**Ultracentrifugal Analyses**—Sedimentation velocity studies on both the hog and the rabbit aldehyde oxidases yielded similar patterns in which the proteins migrated as single symmetrical peaks. When the \(S_{20,w}\) values determined at five protein concentrations between 2 and 13 mg per ml were plotted against protein concentration and extrapolated to zero concentration a value of 14.4 S was obtained for the rabbit liver enzyme. In a similar study for the hog liver enzyme, involving six protein concentrations between 2 and 12 mg per ml, the extrapolated value was 12.5 S. Sedimentation equilibrium analyses of the purified enzyme gave linear plots when the logarithm of the protein concentration was plotted against the square of the distance of each fringe from the center of rotation. Thus, by ultracentrifugal criteria both enzymes were homogeneous. Molecular weights of 275,000, 270,000, and 269,000 were calculated from data collected from sedimentation equilibrium studies of the rabbit enzyme at protein concentrations of 0.3, 0.5, and 1.0 mg per ml. At protein concentrations of 0.25 and 1.0 mg per ml, sedimentation equilibrium studies of the hog enzyme gave molecular weights of 275,000 and 261,000, respectively. A partial specific volume of 0.725 was used in making these calculations. It was calculated from the amino acid composition of the rabbit aldehyde oxidase.

**Absorption Spectra**—Additional criteria of the purity of the hog and rabbit enzymes are their spectra with clearly defined absorption maxima. The spectra of the two proteins are presented in Figs. 1 and 2. The ultraviolet portions of the spectra are typical of proteins in general. The visible portions were

<table>
<thead>
<tr>
<th>Step</th>
<th>Aldehyde oxidase</th>
<th>Xanthine oxidase</th>
<th>2-Pyridone/4-pyridone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Clarified homogenate</td>
<td>562</td>
<td>23,300</td>
<td>24,900</td>
</tr>
<tr>
<td>2. Heat treatment</td>
<td>463</td>
<td>22,300</td>
<td>14,080</td>
</tr>
<tr>
<td>3. (NH(_4))(_2)SO(_4), 0.30-0.45</td>
<td>66.0</td>
<td>25,800</td>
<td>4,710</td>
</tr>
<tr>
<td>4. Acetone, 0.40-0.47</td>
<td>54.7</td>
<td>18,200</td>
<td>1,420</td>
</tr>
<tr>
<td>5. Alumina Cy</td>
<td>18.0</td>
<td>11,660</td>
<td>730</td>
</tr>
<tr>
<td>6. DEAE-cellulose</td>
<td>5.4</td>
<td>11,740</td>
<td>97.1</td>
</tr>
<tr>
<td>7. Preparative electrophoresis</td>
<td>1.5</td>
<td>1,610</td>
<td>14.7</td>
</tr>
</tbody>
</table>

* Absorbance change per min.
WAVELENGTH

FIG. 1. Absorption spectrum of rabbit liver aldehyde oxidase. The enzyme, 0.4 mg per ml, was in potassium phosphate buffer, 925 mM, pH 7.8. The same buffer served as blank. The inset is a partial spectrum of a different preparation of enzyme at a concentration of 6.13 mg per ml in deionized water.

300 400 500 600
WAVELENGTH (μm)

300 400 500 600
WAVELENGTH (μm)

FIG. 2. Absorption spectrum of hog liver aldehyde oxidase. The enzyme was in potassium phosphate buffer, 66.7 mM, pH 7.5 at a concentration of 1.57 mg per ml.

qualitatively the same as the spectrum reported by Rajagopalan and Handler (29) for the rabbit enzyme. The shoulder at 350 nm which was present in their enzyme preparations was resolved into the broad and clearly defined peaks in the spectra shown in Figs. 1 and 2. The $E_{350}/E_{280}$ ratio of the purified rabbit aldehyde oxidase in deionized water was 1.24; its $E_{280}/E_{280}$ ratio was 5.2 to 5.3. The $E_{280}/E_{400}$ ratio of the purified hog aldehyde oxidase was 1.3 and its $E_{280}/E_{400}$ ratio was 5.2 to 5.8. It is interesting to note that the spectra of both the rabbit and hog aldehyde oxidases are very similar to that reported for milk xanthine oxidase (30).

Electrophoretic Analyses—An additional indication of the purity of the hog and rabbit aldehyde oxidases came from their analyses by polyacrylamide disc gel electrophoresis at pH 8.5 and 9.5 and gel concentrations from 4 to 9%. These studies permitted the conclusion that these preparations were 99% pure, that is, size or charge variant contaminants could represent no more than 1% of the protein in any preparation. Using the disc electrophoretic method of Hedrick and Smith (28), a molecular weight of 270,000 was estimated for the hog enzyme and a molecular weight of 260,000 for the rabbit enzyme. These values are in good agreement with those obtained in the ultracentrifugal studies reported above.

Both the hog and the rabbit enzymes have an ability to spontaneously polymerize into higher weight species. Aggregation was first suggested by the trailing of the enzymes on column chromatography, and positively demonstrated when previously homogeneous enzyme preparations which had been stored for 2 weeks at -15° were subjected to disc gel electrophoresis. In the case of the rabbit enzyme, disc gel electrophoresis revealed a series of small polymeric forms (Fig. 3).

By way of contrast, the polymerized hog aldehyde oxidase seemed to be of very high molecular weight since it did not migrate into the gels but collected on their upper surfaces. Polymeric forms of aldehyde oxidase purified from hog liver have been reported by Mahler et al. (31). The aggregation of both the rabbit and hog enzymes appeared to be concentration dependent. Since the aggregation of the rabbit enzyme occurred slowly, it was possible to analyze the size range of the polymeric forms using the method of Hedrick and Smith (28). The molecular weights estimated for the major polymers were 460,000 and 540,000; trace amounts of species having even higher molecular weights were also evident.

Complete reversal as well as the prevention of the polymerization of both enzymes was achieved with thiol reagents. Mercaptoethanol, glutathione, and dithiothreitol were found to be at least partially effective in reversing polymerization of the rabbit enzyme at 0.06 M, 0.0016 M, and 0.0016 M concentrations, respectively. While the addition of low concentrations of thiol reagents gave a slight activation of enzymatic activity, it was found, in confirmation of Rajagopalan and Handler's results (32), that higher concentrations cause a marked inhibition. Cysteine used at a concentration of 0.005 M was found to be effective in preventing and reversing polymerization and giving maximum activation of both enzymes. Concentrations of cysteine as high as 0.05 M, while still preventing or reversing polymerization of the rabbit enzyme, caused a sharp decrease in enzyme activity. Although no specific evidence is available which
bears on the nature of this inhibition, a likely possibility is the
disruption of disulfide bonds essential to enzyme activity. The
activation of the rabbit enzyme is believed to have resulted from
the disruption of polymers less active than the 270,000
molecular weight species. This explanation is, however, in
apparent conflict with the conclusion of Mahler et al. (31) that
all of the polymeric forms of the hog enzyme had identical cata-
lytic properties. Since it is not clear that the polymeric forms
are the same in the two systems, there may be no real inconsis-
tency in the two conclusions.

**Composition**—The flavin component of the rabbit enzyme
was identified as FAD by the increase in fluorescence which
occurs when FAD is hydrolyzed in acid; 2.03 moles of FAD
per mole of enzyme were found. An average value of 7.02 g
atoms of iron per mole of the rabbit enzyme was obtained, Table
III. Both the FAD and iron analyses are in agreement with
the values previously reported by Rajagopalan et al. (1). Since
molybdenum has been implicated in the mechanism of action
of aldehyde oxidase (33, 34), the molybdenum content of several
enzyme samples was determined. The tolueno-dithiol method
of Clark and Aslcy (20) gave values which ranged from 0.66 to
1.14 g atoms of molybdenum per mole of enzyme (Table III).
Those values are below the 2.0 reported by Rajagopalan et al.
(1). The reason for the low values obtained in the present
investigation may lie in the fact that the conditions and pro-
cedures used in the purification of the enzyme closely approxi-
mate methods which have been reported to bring about the
removal of molybdenum from aldehyde oxidase. Mahler et al.
(31) reported the complete removal of molybdenum from hog
liver aldehyde oxidase by dialysis against 0.01 m ammonium
hydroxide and 0.1 m Tris-HCl buffer, pH 8.1. The same in-
vestigators also reported that molybdenum could be reduced to
very low levels when purified santhine oxidase was precipitated
by ammonium sulfate or appropriately dialyzed (30).

**Other Properties**—The pH optima of the two enzymes
was found to be different, 9.0 for the rabbit enzyme and 10.5 for the
hog enzyme.

The $K_m$ of the rabbit enzyme for $N^1$-methylnicotinamide was
found to be $6.6 \times 10^{-4}$ m; the $K_m$ of the hog enzyme was $3.6 \times
10^{-4}$ m. The stoichiometry of the reaction was examined using
the rabbit enzyme. When the course of the reaction was fol-
lowed both spectrophotometrically and using an oxygen elec-
trode, the formation of 17.4 $\mu$moles of the pyridones was ac-
accompanied by the consumption of 16.6 $\mu$atoms of oxygen.
Peroxide was confirmed (1) as a product of the reaction by the
doubling of the rate of oxygen consumption in the absence of
catalase and the protective effect the addition of catalase had on
the enzyme in assay mixtures. The presence of catalase in the
assay mixtures for both purified enzymes significantly increased
the period of time during which linear kinetics could be ob-
tained.

**One Enzyme Hypothesis**—The observation that under a variety
of conditions the 2- and 4-pyridones are produced in constant
ratio by a number of animal species (2) led to the idea that in
*vivo* these pyridones might be produced from $N^1$-methylnicotin-
amide through the catalytic action of a single enzyme. When
genetic variants from the typical pattern of pyridone production
in the mouse were discovered, it became possible to perform a
 genetic test of the hypothesis. It was found that the synthesis
of the 2- and 4-pyridones was controlled by a single autosomal
locus (35, 36). Although kinetic analysis provided evidence
that the variant differed in the structural gene of a single enzyme
(4), the possibility remained that the phenomenon was due to a
single activator or inhibitor affecting two enzymes in a parallel
fashion and the question as to whether one or two enzymes were
involved remained open. Direct proof of the one-enzyme hy-
pothesis required purification of aldehyde oxidase to ho-
mogeneity. This purification has now been accomplished for
the hog and rabbit enzymes and several criteria have been used
to verify the purity of the final preparations. The one-enzyme
hypothesis has been proven by the finding that the homogeneous
rabbit and hog liver aldehyde oxidases catalyze the oxidation of
$N^1$-methylnicotinamide to the 2 and 4 pyridones in the same
ratios (Tables I and II) as had previously been observed in *vivo*
(2).

The relative constancy of the pyridone ratios throughout the
purification of the rabbit enzyme gives added weight to this
conclusion since it implies the absence of other enzymes having
the potential for oxidizing $N^1$-methylnicotinamide to the 2-
and 4-pyridones. There was some fluctuation in estimates of
the pyridone ratio for the rabbit enzyme. This was due to the
small amounts of the 4-pyridone relative to the 2-pyridone pro-
duced in the rabbit system and the large effects on the ratio of
small errors in the determination of the 4-pyridone. Their ratio
was, within the limits of experimental error, constant at each
step in the purification procedure. The ratio determined using
several different purified enzyme preparations ranged from 109
to 208 with an average of 168. Xanthine oxidase is the only
other enzyme known to be capable of catalyzing the oxidation
of $N^1$-methylnicotinamide. It is clear from the data presented
in Table I that xanthine oxidase did not contribute significantly
to the production of the 2-pyridone under the assay conditions
used in this investigation of the rabbit enzyme. The loss of
more than 90% of the xanthine oxidase activity in the acetone
fractionation step did not result in a change in the pyridone
ratio.

The situation for the hog liver enzyme was rendered less clear
due to the choice of an assay pH value of 9.5. Although greater
sensitivity was gained, a sacrifice was made in that at this pH
value hog liver xanthine oxidase can play a significant role in
the oxidation of $N^1$-methylnicotinamide (3). The activity of
xanthine oxidase was only partially controlled by the presence
of allopurinol in the assay mixtures. Thus, upon removal of
xanthine oxidase in the course of purification (Table II), the
pyridone ratio fell from 4.4 to 3.8. The pyridone ratio of the
purified enzyme was the same in the presence or the absence of

### Table III

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Composition Fe</th>
<th>Specific activity</th>
<th>Composition Mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.6</td>
<td>0.06</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>8.4</td>
<td>1.14</td>
<td>1.02</td>
</tr>
<tr>
<td>3</td>
<td>7.8</td>
<td>1.002</td>
<td>1.14</td>
</tr>
<tr>
<td>4</td>
<td>7.4</td>
<td>0.93</td>
<td>1.77</td>
</tr>
</tbody>
</table>

*Composition:* The flavin component of the rabbit enzyme was identified as FAD by the increase in fluorescence which occurs when FAD is hydrolyzed in acid; 2.03 moles of FAD per mole of enzyme were found. An average value of 7.02 g atoms of iron per mole of the rabbit enzyme was obtained.
allopurinol. Since the value of 3.8 is the same as the ratio of the pyridones found in hog urine (2), it is presumed that xanthine oxidase plays essentially no role in the oxidation of N1-methylnicotinamid e in vivo.

With the exception of the cases of man and the rat (13) all available evidence supports the notion that aldehyde oxidase is the catalytic agent responsible for the synthesis of both the 2- and 4-pyridones in vivo.

Pyridone Ratio of N1-substituted Analogue—The rabbit and hog aldehyde oxidases were tested for their ability to catalyze the oxidation of the N1-propyl analogue of N1-methylnicotinamide to the respective 2- and 4-pyridones. The ratio was determined on the chromatographically separated products. For both enzymes, the ratios of the pyridone products formed were clearly dependent on the nature of the group with which the nicotinamide was substituted (Table IV). The pyridone ratio fell with the introduction of the larger N1-propyl group, with its increased ability to sterically hinder attack by hydroxyl ions at the ortho position to the ring.

Mechanism—Since a variety of electron acceptors will function in place of oxygen in the oxidation of N1-methylnicotinamide (2, 5, 37), one may presume that the oxidative role of the enzyme with respect of N1-methylnicotinamide is acceptance of a pair of electrons, and that water is the ultimate source of the oxygen atoms which finally appears in the pyridone products. Using these premises and assuming a role of the known tendency of N-substituted nicotinamides to form pseudobases (38), the mechanistic working hypothesis presented in Fig. 4 is proposed. The essential features of the mechanism are attack by a hydroxyl ion either ortho or para to the ring nitrogen forming the respective pseudobases. Presumably pseudobase formation at the position between the ring nitrogen and the carboxamide group is prohibited by the enzyme environment, although oxidation at this position is chemically feasible (39). It is presumed that the relative reactivities of the two accessible positions are also a function of the active site environment. The oxidative step is viewed as being identical for both pseudobases involving transfer of an electron pair through FAD to oxygen. The hydroxylated products then rearranged to the pyridone forms.

Examination of the data shown in Table IV can be interpreted as giving preliminary support to the idea that the pyridone ratio is determined by the initial accessibility of the attaching base to the nicotinamide ring. Thus, the ratio of the pyridones fell off dramatically as the small N1-methyl group was replaced by the larger N1-propyl group, with its increased ability to sterically hinder attack by hydroxyl ions at the ortho position to the ring. This bulkier substituent also might have caused a change in the probability of substrate being bound in each of two hypothetical alternate binding conformations. Either steric hindrance on altered binding restrictions could have produced a lessened likelihood for the formation of the 2-pyridone and lower pyridone ratios. These two parameters also provide a simple explanation for the variation in the pyridone ratio's characteristic of the mammalian species (2). The determination of accessibility in each case would presumably be a function of both enzyme environment around the active site and the nature of the N1-substituent.

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REFERENCES

Fig. 4. Proposed mechanism of action of aldehyde oxidase.

Table IV

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hog</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1-Methylnicotinamide</td>
<td>3.8-4.1</td>
<td>100</td>
</tr>
<tr>
<td>N1-Propyl nicotinamide</td>
<td>0.64</td>
<td>4.1</td>
</tr>
</tbody>
</table>

2-Pyridone to 4-pyridone ratios

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